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Cloning and characterization of spike and floral meristem identity genes in miracle wheat (*Triticum turgidum* var. *mirabile*)

Damla Güvercin[1*], Yaşar Karakurt[2]

[1]Suleyman Demirel University, Department of Biology, 32260, Isparta, Turkey

[2]Isparta University of Applied Science, Department of Agricultural Biotechnology, 32260, Isparta, Turkey

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One of the most common features of plant species belonging to the Gramineae family is to develop normal (unbranched) spikes. However, this unusual spike morphology places *Triticum turgidum* var. *mirabile* into a very special position among all the species of Gramineae. *T. turgidum* var. *mirabile* is known as 'miracle-wheat' with branched heads. In this study, the full length nucleotide sequences of the three genes *APETALA 1* (*AP1*), *APETALA 3* (*AP3*), *PISTILLATA* (*PI*) which are responsible for the formation of the floral meristem identity genes were isolated and characterized. In this context, the partial cDNA fragments obtained by PCR with degenerate primers were analyzed via 3' and 5' rapid amplification of cDNA ends (RACE) analysis to obtain full length genes called *TmAP1*, *TmAP3* and *TmPI*. After cloning and sequencing *TmAP1*, *TmAP3* and *TmPI* genes were found to consist of 1256, 1223 and 1031 nucleotides, respectively. The open reading frames (ORFs) of *TmAP1*, *TmAP3* and *TmPI* encode predicted proteins of 323, 276 and 252 amino acids with a length of 975, 831 and 759 nucleotides, respectively. The predicted proteins of all three genes contained the MADS domain, while the other regions were more variable and less conserved. In comparison to the protein homologs determined for other plants such as *Arabidopsis*, the deduced TmAP3 and TmPI proteins did not have the conserved euAP3 and PI motifs. Southern blot analyse showed that *TmAP1* and *TmPI* genes had single copies and *TmAP3* gene had 2 copies in the *T. turgidum* var. *mirabile* genome.

Keywords: Miracle wheat, *Triticum turgidum* var. *mirabile*, flowering genes, *APETALA1*, *APETALA3*, *PISTILLATA*

Introduction

In order to understand the molecular stages behind flower development, studies were carried out in model organisms such as *Arabidopsis*[1], *Antirrhinum majus*[2-3] and *Oryza sativa*[4]. An analysis of the flower homeotic mutants of *Arabidopsis* and *Antirrhinum* in 1991 yielded an ABC flower model showing that the flower organs including sepal, petal, stamen and carpal were formed by three groups of genes combination[5].

In the ABC model, three gene classes direct the formation of four distinct types of flower organs. According to the ABC model, *APETALA1* (*AP1*) (from the *SQUA*- like gene group) and *APETALA2* (*AP2*) genes expressed in the first whorl provide sepal formation the combined expression of *APETALA3* (*AP3*; from the *DEF*- like gene group) and *PISTILLATA* (*PI*; from the *GLO*- like gene group) genes expressed in the second whorl forms petals. The expression of these genes allows the formation of petals. The co-expression of B and C function *AG* genes in the third whorl leads to the formation of stamens, while the expression of the *AG* gene in the fourth whorl only provides the formation of carpel[6].

In view of the structure of the complex proteins that bind to the regulatory regions of the genes, ABC model has been expanded to the ABCDE model with A specifying sepals, A-B-E petals, B-C-E stamens, C-E carpels and D ovules[7]. Among these genes, *AP1* gene is a typical class A floral organ identity gene and is a MIKC-type MADS box transcription factor that regulates developmental procedures in plants[8-9]. This gene plays an important role in the identification of sepal and petal identity from flower organs after the formation of the flower meristem[10]. *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) are the class B flower organ identity genes and are necessary for the formation of petal and stamen.

Spike morphology differs in 'miracle-wheat'[11]. Tetraploid 'miracle-wheat' display non-canonical spike branching is replaced by lateral branch like structures that resemble small sized secondary spikes. This leads to a higher spike yield by producing

*Author for correspondence:
damlaguvercin@sdu.edu.tr

significantly more cereals per spike as a result of branch formation in 'miracle-wheat'. In this paper, we isolated and functionally characterized *TmAP1*, *TmAP3* and *TmPI* genes of 'miracle-wheat' by using RT-PCR and RACE analysis methods.

Materials and Methods

Plant Material

Triticum turgidum var. *mirabile* samples were collected at the early stage spike and flower buds from a population near Isparta, Turkey in May 2016. For genomic DNA and RNA extraction, the leaf tissues of young seedlings were germinated and grown under short day (8/16 h) growth conditions at 20±2°C temperature and 51-54% relative humidity.

RNA Isolation and cDNA Synthesis

A modified isothiocyanate method[12] was used for total RNA extraction from the leaf tissues and RNA was quantified spectrophotometrically. We quickly visualized the RNA sample integrity on formal dehyde agarose gel and to remove contaminating DNA from RNA preparations, DNase I treatment was applied with a DNA free kit (Invitrogen). The reverse transcripts of purified RNAs, whose quality and quantity were checked, were obtained with advantage RT-PCR kit (Clontech) following manufacturer's instructions.

Since there is no information available about the flowering and house keeping genes in *T. turgidum* var. *mirabile*, primers were designed to isolate the gene homologues of *AP1, AP3* and *PI*. Conserved amino acid sequences from different plant species were used for degenerate primers design (Table 1). These primers were used in PCR reactions for the amplifications of the genes. The PCR was performed using 4 µl of cDNA as template, 0.2 µM of each of the primers, 0.2 mM of each dNTPs, 1.5 mM $MgCl_2$ and 1.25 U *Taq* polymerase (Invitrogen). Then the samples were placed in a thermocycler (BioRad®) under cycling condition as initial denaturation at the 95°C for 3 min, followed by 36 cycles of denaturation at the 95°C for 45 s, annealing at the 56°C for 45 s and extension at the 72°C for 1 min, final extension was carried out at the 72°C for 10 min. The PCR products were separated on 1.2% (w/v) agarose gel. The expected PCR products (*AP1* 580 bp, *AP3* 420 bp,
PI 330 bp) and directly ligated to the linearized pCR 2.1 TOPO vector as per the manufacturer's protocol (Thermo Fisher Scientific, USA, K4500-01) and transformed into competent *Escherichia coli* DH5α cells. There combinant plasmids were identified and the positive clones were sequenced by the dideoxy method using an ABI3730 automated sequencer (IONTEK, Turkey). Both cDNA sequences and deduced amino acid sequences were BLAST searched. Homology search was carried out online at the nucleotide level with BLASTn and at amino acid level with BLASTp (http://www.ncbi.nlm.nih.gov/blast/).

Table 1 — Primers used in this study

Genes		Oligonucleotide of Primers
APETALA1	F	5'GGTAGRGTNCARYTGAAGMG 3'
	R	5'GAGTCAGDTCVAGMTCRTTCC 3'
APETALA3	F	ATGGGDMGDGGRAARRTHGA
	R	TTBGGCTGMATHGGYTGVAC
PISTILLATA	F	GGMAAGATHGAGATMAAGMRG
	R	GCAGATTKGGCTCVAWNGG
RACE-*AP1*	3P	GGATCCGTCTTGATGCGTTACGTTCAC
	5P	AAGCTTGCCAGGACGATGGTGAATGCC
RACE-*AP3*	3P	GCAGGATCCCTGTTACCATAGCGAA
	5P	CTTGAAGCTTGTTCTGTATGAAACACT
RACE-*PI*	3P	GGGATCCCTTCTAGGCCTAAGCAA
	5P	GTAAGCTTGGTCTGTAGAGAGTCC

Amplification of the *TmAP1, TmAP3* and *TmPI* Genes Using RACE Analysis

For full length cDNA synthesis, cDNA sequences of *TmAP1*, *TmAP3* and *TmPI* were used to design gene specific primers according to the sequenced 3` ends. These gene specific primers were also listed in Table 1. Rapid Amplification of cDNA Ends (RACE) analysis was performed to obtain the full length sequences of the genes using SMART RACE-cDNA amplification kit (Clontech, 634923).

Nucleotide Sequence and Bioinformatic Analysis

The nucleotide and deduced amino acid sequences of *TmAP1*, *TmAP3* and *TmPI* were used for BLAST on GenBank / EMBL database. In order to convert nucleotide sequences into protein sequences a translation tool (http://www.fr33.net/translator.php) was used. BioEdit[13] and ClustalW (version 2.1)[14-15] methods were used to align nucleotide and protein sequences. For phylogenetic analysis, neighbor joining trees with 1000 bootstrap replicates were performedusing MEGA6 software[16].

Southern Blot Test

Southern blot analysis was performed according to Sambrook *et al*[17] in order to determine how many copies of the target genes were available in *T. turgidum* genome. For this purpose genomic DNA was isolated

from young leaves of *T. turgidum* by cetyltrimethylammonium bromide (CTAB) method[18]. Genomic DNA was cut with *Hin*dIII, *Eco*RI, *Hin*dIII and *Bam*HI (MBI Fermentas) and transferred to a positively charged Nylon membrane. The DNA probes were labeled with DIG using the DIG DNA labeling kit (Roche Diagnostics). The detection was performed using the CDP-star kit (Roche Diagnostics) protocol.

Gene Expression Analysis with RT-PCR

Young root, stem, leaf and various stages of spike tissues of the *T. turgidum* var. *mirabile* were used for gene expression assays with qRT-PCR. One microgram of total RNA from each sample was used for the reverse transcription reaction. Triplicate quantitative assays were performed on 1 µl of the reverse transcription product using an Applied Biosystems 7500 fast real time PCR system (Applied Biosystems, USA). Real time PCR experiments were performed according to the instructions of the HotStart- IT SYBR green (Thermo Fisher Scientific, USA, 75762). Reactions were set up with QuantiTect probe PCR master mix (HotStart *Taq* DNA polymerase, QuantiTect probe PCR buffer, dNTP mix, 8 mM $MgCl_2$ and specific primer pairs labeled with TaqMan probes for three distinct target gene regions) and approximately, 1 µg of each diluted cDNA (1/10) and RNA free water. The cycling conditions were as follows: 1st cycle at 94°C for 15 min as initial denaturation; 42 cycles of 95°C for 15 s (denaturation), 52°C for 30 s (annealing) and 72°C for 30 s (extension). RT-PCR of the house keeping gene *β-Actin* was used as an internal control and to normalize data control using the primer pair: 5'-CAGCAACTGGGATGATATGG-3' and 5'-ATTTCGCTTTCAGCAGTGGT-3'. The values for the mean expression and standard deviation (SD) were calculated from the results of three independent replicates.

Results

Cloning of *TmAP1*, *TmAP3* and *TmPI* cDNAs from *T. turgidum*

In consequence of the 3' and 5' RACE analysis, the full length cDNA nucleotide length of *TmAP1* was determined as 1256 nucleotides. In the sequence, 5'-UTR (un-translated region) consisted of 52 base pairs (bp), protein coding region 975 bp and 3'-UTR region 212 base pairs. The polyadenylation signal and the poly (A) tail of 17 bp were obtained in the 3'UTR of the gene and the protein encoded by *TmAP1* was 323 amino acids in length. The estimated molecular weight and isoelectric point of *TmAP1* protein were 36.63 kDa and 9.38, respectively. The number of acidic amino acids (Asp + Glu) in the sequence was 32 and the number of basic amino acids (Arg + Lys) was 46. Because of these properties the protein shows a basic property. The length of full cDNA sequence was 1223 bp for *TmAP3* and contained a 831 bp ORF encoding a predicted polypeptide of 276 amino acids. The sequence contained a 99 bp 5'-UTR, a 281 bp 3'-UTR and a 12 bp poly(A) tail. The predicted molecular weight (MW) of the TmAP3 protein was 31.66 kDa and its theoretical isoelectric point (PI) was 11.09. The number of acidic amino acids (Asp + Glu) found in the sequence was 16 and the number of basic amino acids (Arg + Lys) was 52. Therefore, the protein showed a basic property. As a result of RACE analyses, the full length cDNA nucleotide sequence of *TmPI* gene was 1031 bp. *T. turgidum PI* gene (*TmPI*) had a 59 bp 5'-UTR, a 759 bp ORF encoding a protein of 252 amino acids in length, a 197 bp 3'-UTR region and a 16 bp poly(A) tail. The molecular weight of deduced *TmPI* protein was 28.38 kDa and its isoelectric point was 9.63. The predicted proteins of these three cDNAs showed high homology with the sequences of other MADS-box proteins (Fig. 1). The

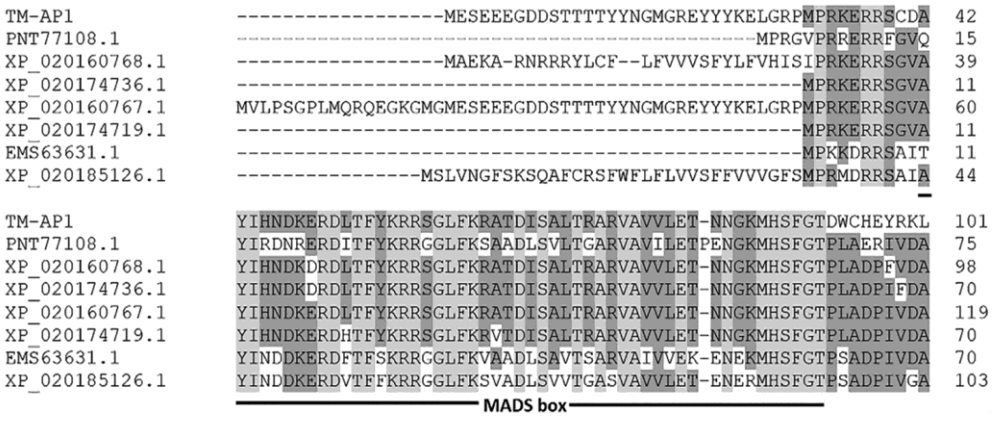

Fig. 1 — Alignment of the TmAP1 protein sequence with some sequences in the GenBank. MADS domain is shown with a solid black line.

alignment of *TmAP1*, *TmAP3* and *TmPI* with other MADS-box proteins showed that they shared highly conserved sequences, mostly in the MADS-box regions. The C and I terminal domains were the least conserved domains.

Amino Acid Sequence Analysis, Phylogenetic Analyses and the Copy Numbers of Genes in 'Miracle-Wheat'

TmAP1 protein showed high similarity to the genes belonging to the *Apetala1* family of proteins (Fig. 1). *TmAP1* was a MADS box transcription factor and was seen to have MADS domain. The MADS domains of the aligned proteins were highly conserved while the I, K and C terminal domains were more divergent. In the BLAST analysis, the *AP1* protein obtained in this study showed a high degree of similarity to its homologues in different plant species. There was a similarity rate of about 32-99% between the TmAP1 protein and about 100 different MADS-box and AGL62 proteins present in the GenBank database. The full length predicted protein sequence showed 99 and 98% amino acid sequence similarity to MADS-box and flower homeotic proteins from *T. spelta* and *Aegilops tauschii*, respectively.

The neighbor joining method (1000 boostrap) was used in the MEGAX package program[16] to determine the phylogenetic relationships among proteins encoded by the isolated *TmAP1*, *TmAP3* and *TmPI* with other MADS box proteins. Approximately, 100 different *AP1* like proteins in the GenBank database had a 28-99% homology ratio to the TmAP1 protein. *T. turgidum* APETALA1 (TmAP1) protein sequence showed the highest similarity to the *AP1* protein sequences of *T. aestivum* (AB012103.3) and *Capsicum annuum* (XP_016542716.1) species. *Solanum lycopersicum* (XP_025886284.1) and *Sesamum indicum* (XP_020547619.1) species also composed of *AP1* like protein sequences. In the other sub group, there were protein sequences of the species including *Lepidium sativum* (AJQ21783.2), *Brassica oleracea* (XP_013589842.1), *B. napus* (XP_022551779.1) and *Raphanus sativus* (XP_018454853.1) (Fig. 2). With the analysis of the Expasy Tool database, *TmAP1* was identified to be ortholog with the *AP1* homologues of *A. tauschii*, *T. aestivum*, *T. urartu* and *Oryza sativa* species.

In the MADS box proteins, the most protected domain is MADS domain. As a result of the BLASTp analysis, it was observed that the protein sequence of *TmAP3* contained the MADS domain (25 amino acids) (Fig. 3). In our study, all the analyzes showed that the *TmAP3* gene was similar to the *Arabidopsis AP3*-like gene.

T. turgidum APETALA3 (*TmAP3*) protein sequence had the highest similarity to the *Cornus officinalis* (JQ753809.1) *AP3* protein. Upon considering the full length amino acid sequences, it was determined that *AP3* proteins of *Sesamum indicum* (NM_001319686.1), *Brassica napus* (XM_022707790.1) and *Raphanus sativus* (XM_018633390.1) species were similar to the TmAP3 protein. Other species with less similarity were *Brassica oleracea* (XM_013747101.1), *Eutrema salsugineum* (XM_024157903.1), *Arabidopsis lyrata* (XM_021024021.1) and *Capsella rubella* (XM_006292532.2) (Fig. 4). Sequence alignment analysis with other *PI*- like proteins has shown that the TmPI protein contained MIKC type MADS (79 amino acids) and K domains (37 amino acids) (Fig. 5).

Ten different sequences including the *T. turgidum PISTILLATA* (*TmPI*) sequence were separated into two main groups (Fig. 6). According to the phylogenetic analysis, the species that showed the highest similarity

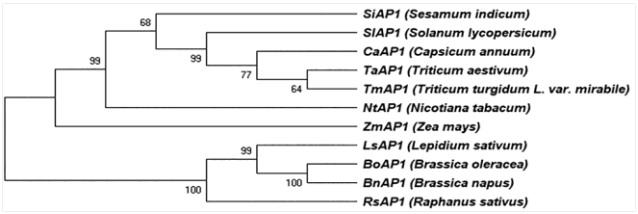

Fig. 2 — Phylogenetic tree of the predicted *T. turgidum APETALA1* (*TmAP1*) homologues proteins and selected AP1 protein sequences obtained from BLASTp analysis at NCBI. The tree was generated by the Neighbor-Joining method using the bootstrap test.

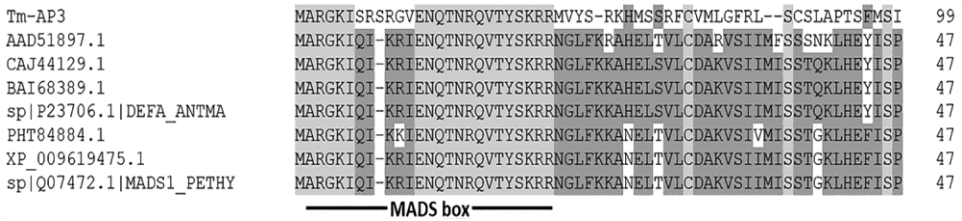

Fig. 3 — Alignment of the *TmAP3* protein sequence with some sequences in the GenBank. MADS domain is shown with a solid black line.

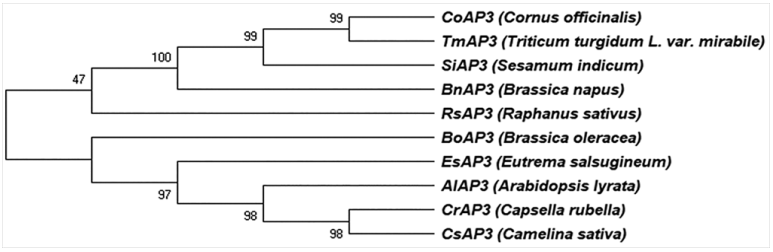

Fig. 4 — Phylogenetic tree of the predicted *T. Turgidum APETALA3* (*TmAP3*) homologues proteins and selected AP3 protein sequences obtained from BLASTp analysis at NCBI. The tree was generated by the Neighbor-Joining method using the bootstrap test.

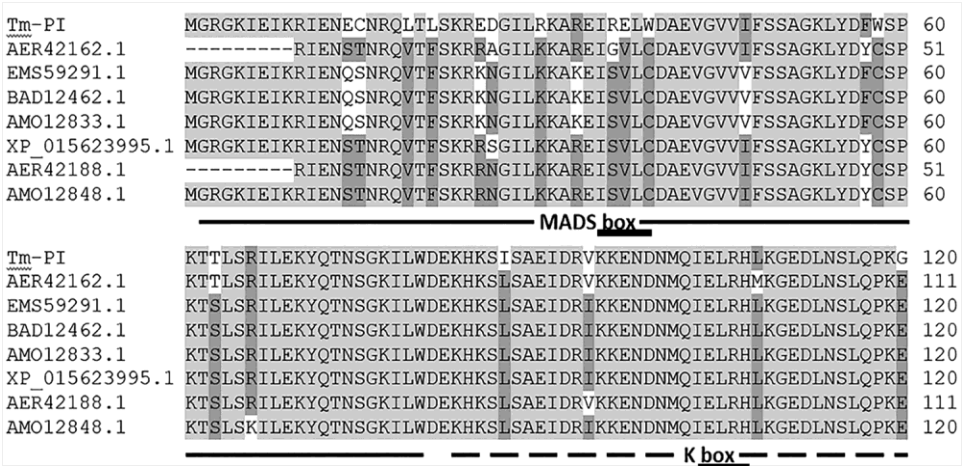

Fig. 5 — Alignment of the *TmPI* protein sequence with some sequences in the GenBank. MADS domain is shown with a solid black line. K domain is shown with a dashed line.

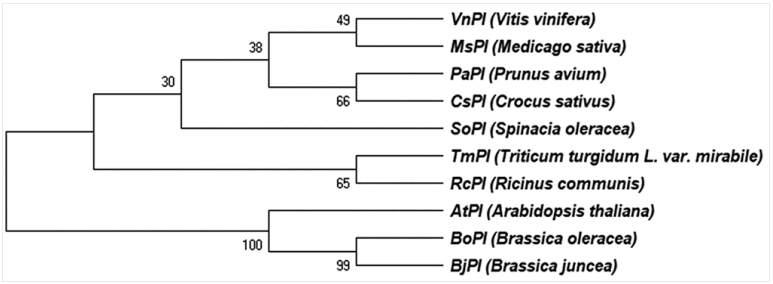

Fig. 6 — Phylogenetic tree of the predicted *T. turgidum PISTILLATA* (*TmPI*) homologues proteins and selected *PI* protein sequences obtained from BLASTp analysis at NCBI.

with *TmPI* protein sequence was *Ricinus communis* (XP_002514306.1). When the full length amino acid sequences were taken into consideration, it was determined that the protein of *Spinacia oleracea* (AAT69985.1), *Crocus sativus* (ABB22780.1), *Prunus avium* (BAT57495.1), *Medicago sativa* (AIT11843.1) and *Vitis vinifera* (NP_001267875.1) species were similar to the *TmPI* protein.

According to Southern blot analysis using digested DNA samples the copy numbers of *TmAP1, TmAP3* and *TmPI* in *T. turgidum* were established (data not shown). It was determined that both genes (*TmAP1, TmPI*) produced one signal by cutting with three different enzymes (*Eco*RI, *Bam*HI and *Hin*dIII). By use a probe derived from *TmAP3* gene, a hybridization pattern with two (2.1 and 2.7 kb) signals after digestion with *Eco*RI enzyme was detected. These findings showed that the *TmAP1* and *TmPI* genes were represented in the *T. turgidum* genome with a single copy and the *TmAP3* gene with 2 copies.

RT-PCR Expression Analysis

To study *TmAP1, TmAP3* and *TmPI* genes biological functions in *T. turgidum* with different developmental processes, we analysed their expression at various stages of spike development (< 4-21 mm in length) young roots, leaves and

stems using RT-PCR. The *TmAP1*, *TmAP3* and *TmPI* showed similar expression patterns in the young spikes. *TmAP1* (AP1/SQUA subfamily) as A function genes; *TmAP3* (AP3/DEF subfamily) and *TmPI* (PI/GLO subfamily) as B function genes of wheat; they were expressed in spikes at three different development stages. To further examine the possible roles of these genes, we performed an expression analysis in various organs at the young stage. Expression of target genes was nearly absent in roots, leaves and stems but was preferentially expressed in young spikes. The expression level of these genes was considerably lower in leaves than in young spikes (*S1*, *S2* and *S3*). Expression of *AP1* was detected in all stages of spike (*S1*, *S2* and *S3*) however, expression level of AP3 and PI was higher in *S2* and *S3* stages, especially (Fig. 7).

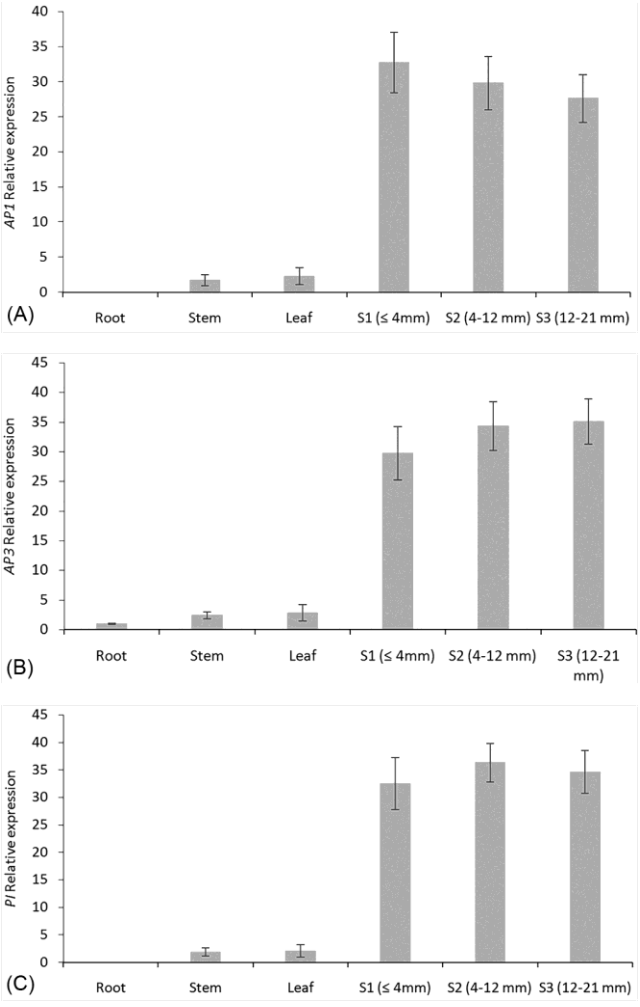

Fig. 7 — Real-time RT-PCR of the three genes. S1–S3 spikes at three development stages (starting with spike of < 4 mm and until complete heading).

Discussion

Flower organ identity genes have been identified as flower homeotic mutants. The flowering organ identifying genes generally encode homeotic transcription factors, which enable the expression of genes of interest leading to the flowering of the plant and formation of flower organs. These transcription factors determine where specific structures develop. Such genes function as development related keys that activate the entire genetic program for a given structure. Therefore, homeotic genes give flower organs their identity. Although majority of studies on MADS box genes are in dicotyledons whereas, studies in monocotyledons had been performed only in rice[19-21], maize[22] and orchids[23-25]. Results obatined from previous studies showed that most of the dicots and monocots other than the *Triticum* genus, *AP1* gene belongs to the *AP1/SQUA* subclass and the protein size encoded varies between 236 - 252 amino acids. Although the *TmAP1* gene consisted of 323 amino acids encoded from the nucleotide sequence, the *AP1* gene isolated from *T. aestivum* has been identified to synthesize 244 amino acids in length[26]. The MADS domain structure of the TmAP1 protein and the predetermined AP1-like protein sequences in plants of the *Triticum* species have a high structural similarity.

Flowering is a phase of transition from vegetative to generative stage in plants and is controlled by vernalization, photoperiodism and genetics[27]. The time of spike in cereals such as barley and wheat is controlled by flowering genes and environmental conditions. The vernalization required to accelerate spike formation is controlled by the genes *VRN1*, *VRN2* and *VRN3*[28]. In the gene mapping studies conducted in *Triticum* genus, it was determined that *VRN1* gene has high similarity to *AP1/FUL* gene found in *Arabidopsis*[29]. However, in the hexaploid wheat species the *VRN1* gene shows high sequence similarity with the *WAP1* (wheat *AP1*)[30-31] or *TaVRT-1* gene[32]. Wheat *AP1*-like *WAP1* gene is also called *VRN1*[32-33]. Similar to our findings, the *WAP1* gene was found to be highly similar to the *VRN1* gene in *T. monococum* and it was found to be orthologous. The *TmAP1* gene obtained in our study which supports this hypothesis has shown a high degree of similarity with the vernalization (*VRN*) genes in different *Triticum* species. The protein encoded by the *TmAP1* gene was highly similar to the *Agamous* like (*AGLG*) MADS-box proteins (59-68%). Our results showed that the wheat *VRN1* gene and the *AP1* and *AGLG*

genes were linked and no differences were observed between the coding regions of these genes and the *VRN1* allele except for the three deletions in the promoter region of *AP1*. According to the phylogenetic relationship dendrogram, the TmAP1 protein sequence had a large sequence similarity (42-99%) with other *AP1* homologues found in NCBI. In our study, all analyzes performed with bionformatic tools showed that the *TmAP1* gene was an *AP1*-like gene.

Southern blot analysis of the *TmAP1* gene revealed that the gene was represented by a single copy in the *Triticum* genome. Similarly, in the study conducted with the rice genome the *AP1* homologue genes *FRMADS6* and *FRMADS7* have been shown to be represented with single copies[34]. In *T. aestivum* it was found to contain a single copy of the *WAP1* gene by cutting the genome with *Eco*RI and *Bam*HI.

In angiosperms, MADS box genes, which function as class B genes are composed of *Anthirrium majus* / *Arabidopsis DEFICIENS* / *APETALA3* (*DEF* / *AP3*) and *GLOBOSA*/ *PISTILLATA* (*GLO/PI*) genes. It is predicted that gene duplications and frame shift mutations in the region encoding the C domain may be effective in the formation of different MADS-boxed gene groups in plants[35-36]. As a result of these mutations and duplications, new motifs have been formed in C domain and these highly conserved regions have introduced their unique functions to proteins. The motifs encoded by B-class MADS box genes were determined as PI, plaeo AP3, euAP3 and TM6[37]. All B-class genes (*AP3* and *PI*) originate from a single ancestral gene as a result of multiple gene duplications[38-43]. It is thought that PI and paleo AP3 motifs are at the C-terminal end and are the ancestors of class B genes. As a result of a second duplication of paleo AP3 line, euAP3 (real AP3) and tomato MADS 6 (TM6) motifs were formed[44]. The bioinformatics analyzes of the predicted proteins of the obtained *TmAP3* and *TmPI* genes showed that these were non-motif B-class MADS-boxed genes. The *AP3* cDNA isolated from *Fagopyrum esculentum* contain a 1090 bp ORF, which encodes a 219 amino acid polypeptide[45]. The cDNA of the *AP3* gene isolated from the orchid plant is 942 bp and encodes a protein containing 204 amino acids. Although there are sequence similarities in this type of *AP3* gene as in *TmAP3*, four of the gene motifs of class B at the C terminal end could not be detected. The 681 bp ORF was predicted to encode a 226 amino acid protein in *Vitis vinifera AP3* (*VvAP3*)[46]. The *PtAP3* cDNA of *Populus tomentos* is 717 bp and the protein encoded is 238 amino acid[47]. The determination that *TmAP3* encodes a protein of 276 amino acids indicates a significant difference in size from other *AP3* homologues. The fact that these differences are determinative in the 3D structure of the protein supports the hypothesis that the related changes are likely to affect the character of the protein and that *TmAP3* may be one of the genes likely to cause multiple spike formation. In the Southern blot analysis of the genome of *B. napus* (canola) three copies of the *AP3* gene were found[48]. In the genome of the orchid plant there were many copies of the *AP3* gene. Similar to our findings, the *AP3* gene was detected as a single copy in the *Crocus sativus* genomic DNA with *Bam*HI and *Hin*dIII enzymes and two copies with *Eco*RI enzyme[49]. This result can be explained with two possible hypotheses. First, the cloned sequence may represent two different alleles of a single genomic locus. This scenario assumes that no part of the genomic DNA has the *BamHI* and *Hin*dIII regions recognized by the probe and that *Eco*RI cuts from a hypothetical intron of *TmAP3*. The second alternative hypothesis may be that the two sequences represent two different genomic loci that can be distinguished by the different *Eco*RI restriction models.

It was determined that the TmPI protein sequence predicted by phylogenetic analysis was similar to PI-like sequences. As a result of sequence alignment with other PI-like proteins the TmPI protein was found to be a transcription factor containing the MADS and K domains (Fig. 5). The proteins encoded by the PI-like genes were determined to have a common PI motif (MPFxFRVQPxQPNLQE) composed of 19 amino acids at the C-terminal end (Stellari *et al*, 2004). In our study, it was determined that there was no PI motif at the C-terminal end of TmPI. Studies have shown that PI motif is not essential for the function of PI-like proteins. PI-like proteins with no PI motifs were also found to be functional in the formation of the petal and stamen identity[50-52]. Similar to our study, the *PsPI* protein isolated from *Pisum sativum* was also found to have no PI motif but the protein was functional and also played a key role in flowering (Berbel *et al*, 2005). It was determined that PI-like genes of the *Medicago truncatula* species did not have a PI motif and this did not cause any changes in the flower morphology. These findings investigate that C-terminal domain is not required for the activity of class B proteins

(Benlloch *et al*, 2009). As a difference, PI-motif proteins have been reported to bind to CArGx boxes more strongly than the proteins without PI-motifs[53].

The *VvPI* gene isolated from the *Vitis vinifera* is 639 bp and encodes a protein with 212 amino acids in length (Poupin *et al*, 2007). The *PI* gene found in *Paeonia lactiflora* is 890 bp long and encodes a protein of 208 amino acids[54]. *T. aestivum WPI* gene contains 928 nucleotides and encodes a protein of 208 amino acids. To support our findings the *WPI* protein was found to be orthologous with PI-like proteins in rice and corn[55]. Two *PI* genes which isolated from *Lilium longiflorum* (*LMADS8* and *LMADS9*) were identified and the protein encoded by one of them was 29 amino acids shorter and did not contain PI motif (Chen *et al*, 2011). Similarly, two different *PI* gene homologs were isolated in *Lotus japonicus* and named *LjPIa* and *LjPIb* was 30 amino acids shorter than the other *PI* homologs and *LjPIa* and did not contain PI motif but both had an active role in the formation of petal and stamen[56]. The protein encoded by *Alpinia oblongifolia PI* gene (841 bp) was 208 amino acids in length. Similar to our findings the MADS and K domain regions of the predicted amino acid sequence of the *AoPI* gene were conserved. Supporting our findings in the amino acid sequence of the *Crocus sativus PI* gene the MADS and K domains were conserved at a higher rate than the others (C and I)[57]. Similar to our study in the *PI* gene of *Antirrhinum majus* there was a K domain in the center of the protein coding region in addition to the MADS box. The K domain encoded a protein that resembled to the amphipathic helical region of the keratin proteins responsible for helical formation[58-60]. Zhang *et al*[61] identified 5 different *PI* gene (*PaPI1-5*) homologues from *Platanus acerifolia* and the K domain was effective in the hetero-dimerization of *PI* and *AP3* but its absence did not cause a negative effect on flower development.

The genomic Southern blot analysis showed that there were 1-2 copies of *PI* gene in *Asparagus officinalis* genome[62]. It has been reported that with the use of four restriciton enyzmes (*Bgl*II, *Hin*dIII, *Eco*RV, *Eco*RI) in *Phalaenopsis equestris* and the use of two restriction enzymes (*Dra*I and *Hin*dIII) in orchid plant showed that the *PI* gene was available in single copies in their genome[63]. According to this information, as in our study the *PI* gene is generally found as a single copy in the genome.

The expression patterns of the *AP1, AP3* and *PI* genes were analyzed by RT-PCR using gene-specific primer sets at various stages of spike development and vegetative tissues. Interestingly, members in A (*AP1*) and B (*AP3/PI*) function genes were found to be primarily expressed in the spikes which suggests that these genes may be involved in the regulation of spike development[64]. In rice, FDRMADS6 was expressed only in young spikes, no signal was present in vegetative tissues, whereas the transcript of FDRMADS7 was detected not only in young flowers but also in root and shoot tissues and the expression in shoot is weaker, which is notable because no other reported rice MADS-box genes are expressed in vegetative tissues[65]. Expression of *TaAP1* was absent in roots, coleoptiles but was high in all other tissues and floral organs; however its rice ortholog OsMADS14 is expressed only in florescence and developing caryopses[66]. Moreover, Japanese pear A (*AP1/FUL*) subfamily members PpMADS2-1 and PpMADS3-1 were also involved in fruit development and ripening[67]. These findings have shown that homologous MADS-box genes from different plants may play various roles in controlling plant growth and development.

In our study we have demonstrated by compare the nucleotide and amino acid sequences of the *AP1, AP3* and *PI* with the homologues in the literature, mutations and sequence differences. In this context the isolation and characterization of the genes that might give this morphological character to *T. turgidum* var. *mirabile* contributed to the understanding of the molecular mechanisms that led to the formation of multiple spikes and the possibility of introducing this feature to the cultivated wheat crops. The data of *AP1, AP3, PI* gene from *T. turgidum* var. *mirabile* through gene isolation, characterization could be a tool for the manipulation of multiple spikes.

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